Steroids 87 (2014) 1–5

Contents lists available at ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

Differential response of C-type natriuretic peptide to estrogen and dexamethasone in adult bone

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ARTICLE INFO

Article history:
Received 4 November 2013
Received in revised form 14 March 2014
Accepted 16 May 2014
Available online 28 May 2014

Keywords:
CNP
NTproCNP
Cancellous bone
Sex steroids

ABSTRACT

C-type natriuretic peptide (CNP) is crucial in promoting endochondral bone growth in mammals including humans but whether this paracrine hormone participates in maintaining bone integrity in the mature skeleton is unknown. Accordingly we studied changes in plasma and bone tissue CNP in anoestrus adult ewes receiving short term anabolic (estrogen) or catabolic (dexamethasone) treatment for 7 days. CNP and the aminoterminal fragment of the CNP prohormone (NTproCNP) were measured in plasma and extracts of cancellous bone excised from vertebral, iliac, tibial and marrow tissues. Concentrations of CNP peptides were much higher in vertebral and iliac extracts than those of tibial or marrow. Both plasma CNP and NTproCNP increased rapidly after estrogen followed by a later rise in bone alkaline phosphatase. Vertebral and iliac (but not tibial or marrow) CNP peptide content were significantly increased by estrogen. Consistent with a skeletal source, plasma NTproCNP was significantly associated with vertebral tissue CNP. In contrast, bone tissue CNP peptide content was unaffected by dexamethasone despite suppression of plasma CNP peptides and bone alkaline phosphatase. We postulate that increases in trabecular bone CNP reflect new endosteal bone formation in these estrogen responsive tissues whereas reduced plasma CNP peptides after dexamethasone, without change in cancellous bone content, reflects reductions in cortical bone turnover.

1. Introduction

C-type natriuretic peptide (CNP) plays an essential part in postnatal endochondral bone growth by promoting chondrogenesis and growth plate expansion in mammals including humans [1,2]. Although all components of the CNP signaling pathway are present in bone tissue [3], and when activated promote osteoblastic differentiation in cell culture [4], little is known of this paracrine factor's role in the maintenance of skeletal integrity in the adult. Despite numerous studies of the phenotype resulting from genetic modifications in the CNP signaling pathway, loss or gain of bone in adults has not been obvious. However increased bone formation was evident in transgenic mice overexpressing B-type natriuretic peptide [5] and further noted in studies where the clearance receptor (NPR-C) was disrupted [6]. Both bone volume and trabecular thickness in the humerus are reportedly reduced in the lbab mouse – where a spontaneous mutation renders CNP inactive – and are restored when these mice are crossed with animals over-expressing CNP in cartilage tissue [7]. These reports suggesting trophic actions of CNP in bone stand in contrast to recent findings in a family exhibiting constitutive over activity in the CNP receptor (NPRB) where skeletal overgrowth in the adolescent proband was associated with reduced bone mineral density [8]. Clearly further in vivo study is required to assess whether CNP – crucial to endochondral growth – also participates in bone remodeling.

In previous studies in the non-cycling adult ewe [9], estrogen administration was shown to promptly increase the plasma concentration of both the bio active mature form of CNP (CNP 22) and the (presumably inactive) product of proCNP synthesis (aminoterminal proCNP, NTproCNP). This response was associated with a threefold rise in bone alkaline phosphatase (bALP) – suggesting that an increase in CNP production within bone could be a source of the sustained increases in CNP proteins observed in plasma. Also consistent with contributions from skeletal tissues is the response of plasma CNP peptides to dexamethasone which rapidly reduces CNP concentrations and alkaline phosphatase in both growing lambs and adult ewes [10,11]. These previous findings are summarized in Table 1. Postulating that these changes in plasma

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http://dx.doi.org/10.1016/j.steroids.2014.05.007
0039-128X/

Table 1
Plasma responses to steroids in sheep.

	Ewe lambs[a]		Adult ewes	
Analyte	Dexamethasone	Estradiol	Dexamethasone	Estradiol
CNP	Fall [10,11]	Rise [9]	Fall [10]	Rise [9]
ALP	Fall [10]	Rise [9]	Fall [10]	Rise [9]

[a] linear growth in lambs was reduced by dexamethasone and unaffected by estradiol.

concentrations would also be reflected in their bone content, we have now studied changes in CNP peptides in plasma and extracts of bone tissue (vertebra, ilium, and tibia) in adult non cycling adult ewes after 7 days of either the anabolic hormone estrogen or (catabolic) dexamethasone. Because bone marrow tissue is enriched in progenitor cells involved in osteoblastogenesis [12,13] marrow extracts from the tibia were also examined.

2. Materials and methods

Twenty-four healthy Coopworth non-cycling adult ewes (>3 years age, live weight range 41–62 kg) maintained on pasture were randomly allocated (8 per group) to receive estrogen treatment (5.6 mg/kg live weight slow release depot, Compudose 400 implants), dexamethasone (1.5 mg/kg/day) or saline solution administered by daily s.c. injection. Doses and duration of treatment were chosen on the basis of previous observations in adult sheep [9,10]. All animal studies were approved by the Lincoln University Animal Ethics Committee.

Serial blood samples were drawn at −7 days, just before starting the intervention (day 0) and then at day 1, 2 and 7 (just prior to euthanasia) for measurement of plasma CNP, NTproCNP and bALP. Following euthanasia, representative skeletal tissues (iliac crest wedge, lumbar vertebra (L4) and left tibia) were rapidly removed, sectioned longitudinally (sagittal plane), and snap frozen under liquid nitrogen for protein assays. Bone marrow tissue was similarly collected and processed from the left tibia. Tissue samples were then stored at −70 °C until analysis.

2.1. Bone tissue peptide measurements

Samples (0.3–1.5 g) of frozen marrow or cancellous bone (subadjacent to the proximal chondro–osseous junction) were crushed by impact in a stainless steel chamber surrounded by dry-ice, then placed in 10 ml boiling water containing 0.01% triton-X100 for 5 min, acidified with acetic acid, and homogenized prior to extraction on Sep-Pak C18 cartridges (Waters Corporation, Milford, Massachusetts, USA) as previously described for ovine pituitary tissue [14]. Extracts were re-suspended in assay buffer for radioimmunoassay as described below for plasma samples. Results are expressed as fmol/g of tissue.

2.2. Plasma assays

Bone alkaline phosphatase (bALP) concentration was measured using heparinised plasma (Ostase, Access, Beckman Coulter, Fullerton, CA, USA). Plasma CNP concentration was measured by radioimmunoassay as previously described [14]. Limit-of-detection for this assay is 1.0 pmol/L (0.2 pmol/L after sample concentration). Within- and between-assay coefficients of variation of the assay are 4.9% and 8.9% respectively; at 2.1 pmol/L. Plasma NTproCNP concentration was measured as previously described [15]. The detection limit of this assay is 1.2 pmol/L (0.4 pmol/L after sample concentration). Within- and between-assay coefficients of variation are 6.8% and 8.4% respectively, at 14 pmol/L.

2.3. Statistical analysis

All values are presented as mean ± SEM. Data for tissue analytes were analyzed by ANOVA. Plasma analyte data obtained during the treatment were analyzed by repeated measures ANOVA using baseline values (day 0) as a covariate and Bonferroni post hoc adjustment for multiple comparisons. Spearman rank coefficient was used to determine correlations between variables, presented as r values. Statistical significance was assumed when $p < 0.05$.

3. Results

Plasma analytes at baseline (just prior to starting interventions) are shown in Table 2. No significant differences were found among groups.

3.1. Plasma responses to interventions

As shown in Fig. 1, compared with saline, highly significant increases in plasma NTproCNP ($p < 0.001$) and CNP ($p < 0.001$) concentrations were observed within 24 h of commencing estrogen treatment, and were sustained over the 7 day period. Similarly, bALP – slower to respond than CNP peptides – was significantly increased at day 7 ($p < 0.001$). Dexamethasone reduced CNP peptides and bALP (Fig. 1). Compared with saline, plasma NTproCNP concentration was significantly suppressed at day 2 ($p < 0.01$), and remained so at day 7. The decline in plasma CNP during dexamethasone treatment was not statistically significant. However, bALP was significantly depressed by dexamethasone ($p < 0.001$) but, as noted with estradiol, the response was delayed and followed that of NTproCNP.

3.2. Bone tissue CNP peptides

Abundance of CNP (Fig. 2A) and NTproCNP (Fig. 2B) in tissue extracts from lumbar vertebra, iliac crest, tibia and bone marrow are shown for each of the 3 treatment groups in Fig. 2. In saline treated ewes, CNP peptides were relatively enriched in vertebral and iliac extracts compared with extracts from tibia or marrow. CNP abundance in vertebral and iliac tissues was significantly increased by estradiol ($p < 0.01$ and <0.05 respectively). NTproCNP content was increased by estradiol in all 4 tissues but only in iliac tissue was the increase significant ($p < 0.05$). Tibial bone marrow tissue CNP and NTproCNP contents were directly correlated with tibial cancellous bone levels ($r = 0.6$ and 0.74 respectively; $n = 16$, $p = 0.02$ and <0.001 respectively).

In contrast to its suppressive action on plasma CNP forms, dexamethasone had no effect on CNP peptide abundance in any of the four skeletal tissues examined (Fig. 2).

In keeping with contributions from skeletal tissues to circulating NTproCNP levels, a strong association of plasma NTproCNP concentration on day 7 with vertebral CNP content was evident in animals receiving saline or estradiol ($r = 0.66$, $p < 0.005$, $n = 16$, Fig. 3). Associations in iliac tissue ($r = 0.5$, $p = 0.057$) just failed to achieve significance.

Table 2
Plasma baseline values (day 0, mean ± SEM).

	Saline	Estradiol	Dexamethasone
CNP (pmol/L)	0.88 ± 0.05	0.77 ± 0.02	0.96 ± 0.17
NTproCNP (pmol/L)	18.9 ± 1.1	18.7 ± 0.7	18.1 ± 1.0
bALP (μg/L)	7.0 ± 1.1	5.3 ± 0.6	6.5 ± 1.0